REMARKS

Reconsideration is requested.

Claims 1-4, 10-16 and 40-50 are pending.

Claims 2, 3, 5-9, 11, 17-39, 41, 42 and 45, have been canceled, without prejudice. The amended claims recite altered plant characteristics. Support for the revisions may be found throughout the specification, such as on page 15, lines 12-17 and in now-canceled claims 11 and 45. Claims 1, 4, 10, 12-16, 40, 43, 44 and 46-50 will be pending upon entry of the present Amendment. Entry of the present Amendment is requested.

Entry of the present Amendment will at least reduce the issues for appeal by making moot the Section 103 rejection of claims 11 and 45 over Kleinow, Sakamoto and Iida (Plant Journal 2000 Oct; 24(2):191-203). Entry of the present Amendment is requested.

The present Amendment will further at least reduce the issues for appeal by obviating the Section 102 rejection of claims 16 and 50 over Kleinow (Plant Journal 2000, July; 23(1):115-22). Specifically, the cited art does not teach plant cells or plant cells with altered characteristics of the claims. Entry of the present Amendment and withdrawal of the Section 102 rejection are requested.

The Section 103 rejection of claims 1-4, 10, 12-15, 40-44 and 46-49 over Kleinow, Sakamoto (Gene 2000 May 2; 248(1-2):23-32) and Kim (Plant Journal 2001 Feb; 25(3):247-59), is also believed to be obviated by the above amendments. Entry of

the present Amendment and withdrawal of the rejection are requested along with consideration of the following comments.

Kleinow is asserted to teach a yeast host cell having modified expression of a nucleic acid which is 100 % identical to SEQ ID NO:1835 and which would have altered characteristics (suppression of snf4 deficiency in yeast). Sakamoto is asserted to teach that expression of AZF2 is strongly induced by various stresses (including high salt, dehydration and ABA treatment). Kim is asserted to teach that expression of SCOF1 would alter cold tolerance of plants. The Examiner is understood to believe that SCOF1 would be essentially similar to SEQ ID NO:1835.

The combination of cited art would not have made the claimed invention obvious. Specifically, Kleinow teaches, at best, the expression of AZF2 in a yeast cell, and not in a plant cell. The yeast cells described by Kleinow are specific yeast cells, i.e., yeast cells carrying the *snf4*Δ2 mutation (see page 116, right column, results).

The Examiner believes that Kleinow would have taught that expression of AZF2 would suppress snf4 deficiency in yeast. However, as it can be seen from table 1, page 116, snf4 deficiency is only suppressed on 3% glycerol, but not on 2% glycerol (see also page 119, left column, first paragraph), whereas other genes disclosed by Kielnow suppress the snf4 deficiency both on 2% and 3% glycerol. Thus, in view of the teachings of Kleinow, the person of ordinary skill in the art would not have been motivated to use AZF2 in order to increase yield and/or biomass in plants.

Further, Kim fails to teach or suggest the claimed invention. Moreover, Kim would not have motivated one of ordinary skill in the art to have made the presently

claimed invention. The SCOF-1 protein described by Kim is only 42% identical to the protein encoded by SEQ ID NO:1835, Thus, SCOF-1 is not essentially similar to the protein of the present claims which only encompasses proteins which are at least 95% identical to the protein as encoded by SEQ ID NO:1835. Moreover, the document Kim only relates to enhancing cold tolerance.

Further, Sakamoto does not teach or suggest that expression of the protein of the present claims would increase yield. Sakamoto only discloses that that the expression of AZF2 is induced under certain conditions, namely as a response to ABA, high salinity and cold. However, the person of ordinary skill in the art would not have understood from the teachings of Sakamoto that expression of the protein encoded by SEQ ID NO:1835 would increase yield and/or biomass. The observation that a certain gene is inducible under certain condition would not have reasonably suggested that this gene, when expressed in a plant, also alters plant characteristics. For example, the attached Ascenzi et al (Plant Molecular Biology, 1999, 41:159) shows that the over-expression of a DNA binding protein which is ABA and drought inducible does not alter plant characteristics (see page 166, discussion, first paragraph).

The claims, as amended, are submitted to be patentable over the cited combination of art. Entry of the present Amendment and withdrawal of the Section 103 rejection is requested.

The claims are submitted to be in condition for allowance. Entry of the present Amendment and a Notice of Allowance are requested. The Examiner is requested to

contact the undersigned, preferably by telephone, in the event anything further is required to place the application in condition for allowance.

Respectfully submitted,

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Molecular genetic analysis of the drought-inducible linker histone variant in *Arabidopsis thaliana*

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Abstract

Linker histones are ubiquitous structural components of chromatin that have been shown to influence the expression of a subset of genes in diverse organisms. Plants contain a minor linker histone variant that is expressed in most tissues of all organs, and is induced during drought stress. Based on reporter gene analysis in roots, *His1-3* is expressed almost exclusively in emerging secondary roots in unstressed plants, but is primarily expressed in the root meristem and elongation zone of stressed plants. In shoots, expression is higher in younger tissues than older tissues. In order to investigate the function of H1-3, we have generated lines with altered levels of H1-3. Plants expressing an antisense *His1-3* transcript exhibit a greatly impaired induction (5% of wild-type RNA levels during stress) of *His1-3* transcripts in shoots during drought and contain decreased protein relative to wild-type control plants. In plants overexpressing *His1-3*, more H1-3 is bound to chromatin than in unstressed wild-type plants. None of the plants containing these transgenes display phenotypic aberrations or differences in water content during drought stress. Additionally, the expression of several drought-responsive genes is not significantly altered in lines misexpressing *His1-3*.

Introduction

The molecular mechanisms that underlie physiological responses to plant water deficit are beginning to be elucidated. A common approach has been to isolate genes that are induced by dehydration stress in *Arabidopsis*. Many of these genes encode polypeptides of unknown function (e.g. *rab18*, *Rd29A*, *AtDi21*), while some encode products for which a function may be ascribed (e.g. *AtP5CS*, *rd28*) (reviewed by Ingram and Bartels, 1996). Most of these genes are also induced by low temperatures, ABA, high salt, or during seed maturation (Bray, 1994). One of these genes, *Rd29A*, has been used to obtain mutants defective in osmotic stress signal transduction (Ishitani *et al.*, 1997). Research into the regulation of these stress-inducible genes by promoter analysis has revealed several important sequence elements and binding proteins. Notably, the ABA-response element (ABRE) has been identified in many stress-inducible genes. In wheat, transcription of the *Em* gene is regulated by the binding of the basic leucine zipper protein, EMBP-1 (Guiltinan *et al.*, 1990) and by the transcriptional activator, VP-1 (McCarty *et al.*, 1991). It has been shown that linker histone H1 dramatically increases the binding activity of EMBP-1 *in vitro* (Schultz *et al.*, 1996) highlighting the importance of chromatin structure in plant gene regulation.

Linker histones (e.g. H1, H5, H1°) have been shown to bind the linker DNA between nucleosomes as well as the nucleosome core particle itself (reviewed by Zlatanova and van Holde, 1996). The globular domain of linker histones has been shown to bind at an asymmetric position within the nucleosome either away (Pruss *et al.*, 1996) or close to the dyad (Zhou *et al.*, 1998). Binding of the globular domain to the nucleosome characteristically protects 20 bp of linker DNA from nuclease digestion (Allan *et al.*,

1980). This property is shared with the HMG-1/2 and HMG-I/Y chromosomal proteins, and it has been proposed that all of these proteins bind a common linker-histone-binding site with varying affinities (Hill and Reeves, 1997). Linker histones are thought to aid the formation and maintenance of higher-order chromatin structure and repress gene transcription, while HMG proteins are associated with matrix-attachment regions and actively transcribed chromatin.

Linker histones vary in their primary sequence, and most higher organisms contain several distinct subtypes. The function of linker histone variants has been explored by gene knockout and RNA reduction strategies mainly in protists and animals. Deletion of the singular macronuclear linker histone gene in *Tetrahymena* led to a decrease in nuclear condensation and an alteration in the transcription of specific genes (Shen *et al.*, 1995; Shen and Gorovsky, 1996). In *Saccharomyces*, deletion of the unique linker histone resulted in no detectable changes in growth or telomeric silencing in several strains (Escher and Schaffner, 1997; Patterson *et al.*, 1998; Ascenzi, Gantt, and Berman, unpublished observations). However, these unicellular organisms have a relatively simple developmental program, and possess single isoforms of linker histones that are structurally different from those found in organisms exhibiting multicellular organization. Indeed, there is evidence that linker histones play a role in animal development. Developmentally regulated accumulation of somatic linker histones in *Xenopus* causes cells to lose competence to differentiate into mesoderm during embryogenesis. The maternal variant, H1M, does not affect competence (Steinbach *et al.*, 1997). This loss of competence appears to be accomplished by transcriptional silencing of select regulatory genes. The regulation of the mouse mammary tumor virus promoter has been shown to be dependent the presence of histone H1 (Lee and Archer, 1998). This underscores the importance of chromatin structure, and histone H1 in particular, in the regulation of a variety of developmentally important genes. In the single published plant report, overexpression of *Arabidopsis His1-2* in tobacco was shown to perturb normal development (Prymakowska-Bozak *et al.*, 1996).

We isolated a divergent linker histone gene, *His1-3*, that is induced by progressive drought stress but not by rapid desiccation, cold, or high salt (Ascenzi and Gantt, 1997). The accumulation of *His1-3* transcripts precedes that of *Rd29A* under these natural drying conditions. This drought-inducible linker histone subtype appears to be conserved among flowering plants and is analogous to the vertebrate H1°/H5 variants in that it is relatively rich in arginine and may function as a replacement variant. We hypothesize that plants have maintained a distinct drought-inducible linker histone variant to cope with the unique physiological conditions that drought stress imposes. We have explored this hypothesis by introducing sense and antisense transgenes under the control of the CaMV 35S promoter to manipulate the levels of H1-3. In this report, we further characterize the expression of *His1-3* and describe changes in H1-3 content in the chromatin of sense and antisense transgenic plants.

Materials and methods

Plant material

For all experiments, seeds of wild-type *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia and transgenic derivatives of the ecotype were sown on horticultural potting mixture (Metro-Mix 360, Scotts Co., Marysville, OH). Stratification was performed for at least two days at 4 °C. Growing conditions were 16 h light (beginning at 07:00), 150 μE m^{-2} s^{-1} with a light-cycle temperature of 22 °C and dark-cycle temperature of 20 °C. Plants were fertilized once, one week after germination, with Miracle-Grow (Scotts, Port Washington, NY) and otherwise watered with distilled water.

Plasmids and plant transformation

The plasmids used in this study were constructed as follows.

1. *His1-3::GUS*. A translational fusion of H1-3 and β-glucuronidase (GUS) was made by directionally ligating a *Hin*dIII-*Bgl*II fragment containing 3.4 kb of *His1-3* upstream sequence (this includes the 5' UTR and the first four codons) into the site created by a *Hin*dIII-*Bam*HI digest of pBI101.3 (Clontech, Palo Alto, CA).
2. *35S::His1-3AS* Clone 128L6T7, obtained from the Arabidopsis Biological Resource Center, containing a nearly full-length H1-3 cDNA, was digested with *Pst*I and *Sna*BI and this fragment was subcloned into the site created by a *Pst*I-*Eco*RV digest of pBluescript KS− (Stratagene, La Jolla, CA). This clone was then digested with *Sac*I-*Hin*cII and ligated into the *Sma*I-*Sac*I sites of pBI121 (Clontech).

3. *35S::His1-3* Clone 128L6T7 was digested with *Eco*RI-*Bam*H1, and directionally subcloned into pBluescript KS−. This construct was then digested with *Sma*I-*Sac*I and ligated into the corresponding sites of pBI121.

All constructs were introduced into *Agrobacterium tumefaciens* and plants were transformed by vacuum infiltration (Bechtold *et al.*, 1993) as described by van Hoof and Green (1996) except that we infiltrated only the inflorescences without immersing leaves of T_0 plants, and transformants (T_1) were selected with 30 μg/ml kanamycin (Kn). T_1 plants were self-pollinated to generate a T_2 population with the transgenes segregating. Plants from the T_2 generation were self-pollinated and their progeny screened (T_3 generation) for 100% Kn resistance indicating that the plants were homozygous for at least one T-DNA locus. While segregation may be occurring at an unlinked locus, the most thoroughly analyzed lines do not contain two unlinked loci. At this stage, independent transformants were confirmed by Southern blotting. Homozygous lines were used for most analyses.

Stress treatments

Plants were subjected to water stress by progressive drought treatment (Gosti *et al.*, 1995). In these experiments, plants were grown in pairs (one wild-type plant and one transgenic plant) in standard-size cell packs. At 14–16 days after germination, water was withheld from a subset of cell packs. After an additional 6–12 days, plants were either harvested for water content analysis or for extraction of RNA or chromatin protein. For these experiments it was useful to have a rapid means of identifying and then determining the degree of drought stress experienced by plants. Two stages of progressive drought stress in *Arabidopsis thaliana* Columbia were characterized on the basis of both gene expression kinetics (Ascenzi and Gantt, 1997) and phenotypic observations. The 'early' stage occurred five to seven days after withholding water and is characterized by *His1-3* and *rab18* induction, an increase in anthocyanin in new leaves as evidenced by a slightly darker appearance and pigment in the RNA extract, and a slight loss of turgor (85–95% relative water content, RWC). The 'late' stage, occurring 8–12 days after the final watering, is typified by induction of the *Rd29A* and *AtDi21* transcripts, repression of *cab* and *rbcS*, a further increase in anthocyanin, and obvious loss of turgor (less than 85% RWC). Most of the results in this paper are from late-stage plant-pairs. All plants were harvested between 13:00 and 15:00 to minimize any diurnal periodicity in transcript accumulation that has been documented for the tomato *His1-3* homologue (Corlett *et al.*, 1998).

Histological assays

Spatial expression of *His1-3* in stressed and unstressed plants was assayed by GUS reporter gene expression. GUS staining was performed as described by Sieburth and Meyerowitz (1997). Stained tissues were either observed directly or embedded in paraffin and sectioned for microscopic observation.

Molecular methods

DNA and RNA was extracted from whole seedlings and analyzed as described previously (Ascenzi and Gantt, 1997) with the following exceptions: RNA probes were used to detect sense and anti-sense *His1-3* transcripts; Hybond-N (Amersham, Arlington Heights, IL) membranes were used for RNA blots; and high-stringency hybridizations were performed by prehybridizing in the buffer previously described (Gantt and Lenvik, 1991) and by adding 0.2 volume 50% dextran sulfate prior to probe addition.

Proteins and antibodies were prepared and analyzed as described previously (Ascenzi and Gantt, 1997) with the following modification for small tissue samples. *Arabidopsis* seedlings weighing 1.5–3 g were quickly frozen and ground in liquid N_2, and resuspended in 0.4 M sucrose, 10 mM $MgCl_2$, 10 mM Tris pH 8.0, and 5 mM 2-mercaptoethanol containing 1 mM PMSF, 1 mM benzamidine, 0.25 mM TLCK, 50 μg/ml TPCK, 5 μg/ml aprotinin, 20 μg/ml antipain, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, CA), and spun for 2 min in a 4 °C microcentrifuge. Washes were also performed in Eppendorf tubes and the impure chromatin was pelleted through 1.7 M sucrose in a Beckman SW 41 rotor (20 000 rpm) for 15 min. Yields were typically 30–40 μg chromatin per g tissue. Proteins were analyzed by SDS-PAGE or acid urea-PAGE (Spiker, 1980) in a mini-slab electrophoresis apparatus (Idea Scientific, Minneapolis, MN) and transferred to nitrocellulose (Gelman, Ann Arbor, MI) in 0.7% acetic acid in a Trans-blot Cell (BioRad, Hercules, CA). Duplicate gels were stained with Coomassie or silver nitrate.

Physiological measurements

Plant water status was evaluated by estimating relative water content (RWC). This value was obtained by the following calculation: [(fresh weight − dry weight)/(turgid weight − dry weight)] × 100. Plants in late-stage water-stress were harvested by cutting above the cotyledons and excising the leaves with a razor, and then these separated leaves and shoot apices (containing leaf primordia) were immediately weighed. The turgid weight was obtained by rehydrating the tissue for 24 h in the dark (cut surfaces down, e.g. petiole) in humid chambers each consisting of a water-saturated tissue paper in a sealed Petri dish. The excised organs were then quickly blotted dry and reweighed. The tissue was then dried in a 65 °C oven for at least 48 h before measuring the dry weight.

Results

Induction and spatial expression of His1-3

A translational fusion between β-glucuronidase and H1-3, containing 3.4 kb of upstream *His1-3* sequence, was constructed in pBI101.3. This construct (*His1-3::GUS*) was introduced into *Arabidopsis* by the vacuum infiltration method (Bechtold *et al.*, 1993). We recovered a total of nine independent Kn^R transformants. All independently transformed lines expressing the reporter had identical expression patterns. As expected, all lines exhibited increased GUS expression when the plants experienced drought stress (Figure 1) indicating that reporter gene expression can be attributed to putative stress response elements contained within the transgene. However, we observed differences in the staining intensity between lines. As negative and positive controls, we stained wild-type plants and plants expressing GUS driven by the 35S promoter in each experiment. We did not observe staining in the wild type and staining in the *35S::GUS* plant was observed throughout the plant. In addition, the staining intensity in drought-stressed *35S::GUS* did not show an increase relative to unstressed plants.

In stressed and unstressed plants, expression is highest in young leaves and inflorescences (Figures 1A and B). In flowers, expression is consistently observed primarily in the pedicels, calyx, ovary (especially at the junction between the ovary and style) and in anthers (Figures 1C and D). While the expression pattern does not change upon drought stress in shoots, in roots GUS expression appears to be shifted from emerging secondary roots in well-watered plants (Figure 1E) to the meristems of already elongated roots in drought-stressed plants (Figure 1F). The overall level of GUS staining in the root system is also elevated in stressed plants relative to controls.

We also observed tissue-specific developmental regulation of *His1-3* expression. In young seedlings, expression is characteristically found near or at the transition zone, the area at the junction of root and hypocotyl (Figure 1G), in hydathodes, guard cells (Figure 1H), and in trichome bases. In mature plants, developing ovules and silique vasculature leading to the ovules (Figure 1I) exhibit high levels of reporter gene expression. While leaf and flower primordia are most intensely stained, the central meristem has undetectable reporter gene expression, although there is expression in the flank meristem zone (Figure 1J and data not shown). Interestingly, the level of GUS staining increases when plants are grown on 3% sucrose and exposed to a dark period relative to plants grown on 1% sucrose and exposed to darkness (compare cotyledon in Figure 1K with 1L). *His1-3* transcript levels in plants grown on 3% sucrose and exposed to a six hour dark period are increased an average of two-fold compared with similarly treated 1% sucrose controls (data not shown).

Aside from its ability to be drought-induced, the tissue-specific regulation of *His1-3* is distinct from *H1-1* and *His1-2*. *His1-3* is not expressed in the central zone of the shoot meristem in contrast to *His1-1*, and also, *His1-3* expression is evident in hydathodes and in the transition zone (Orcutt and Gantt, unpublished data). However, there is considerable overlap with the two highly expressed variants indicating that the linker histone variants probably co-exist within many tissues.

Plants expressing the His1-3 *cDNA in the antisense orientation contain decreased levels of H1-3 transcripts and protein*

We transformed a construct containing an H1-3 cDNA in the antisense orientation driven by the 35S CaMV promoter (*35S::H1-3AS*) into *Arabidopsis* by vacuum infiltration in two pools of six plants. From a total of 13 Kn^R T_1 plants recovered, 10 had unique Southern blot profiles from analysis of DNA extracted from T_2 plants (data not shown). These independent *35SH1-3AS* lines varied in transgene copy number from one (line ASb7) or two (ASb1 and ASb4), to several dozen (ASa2 and ASb8) (Table 1).

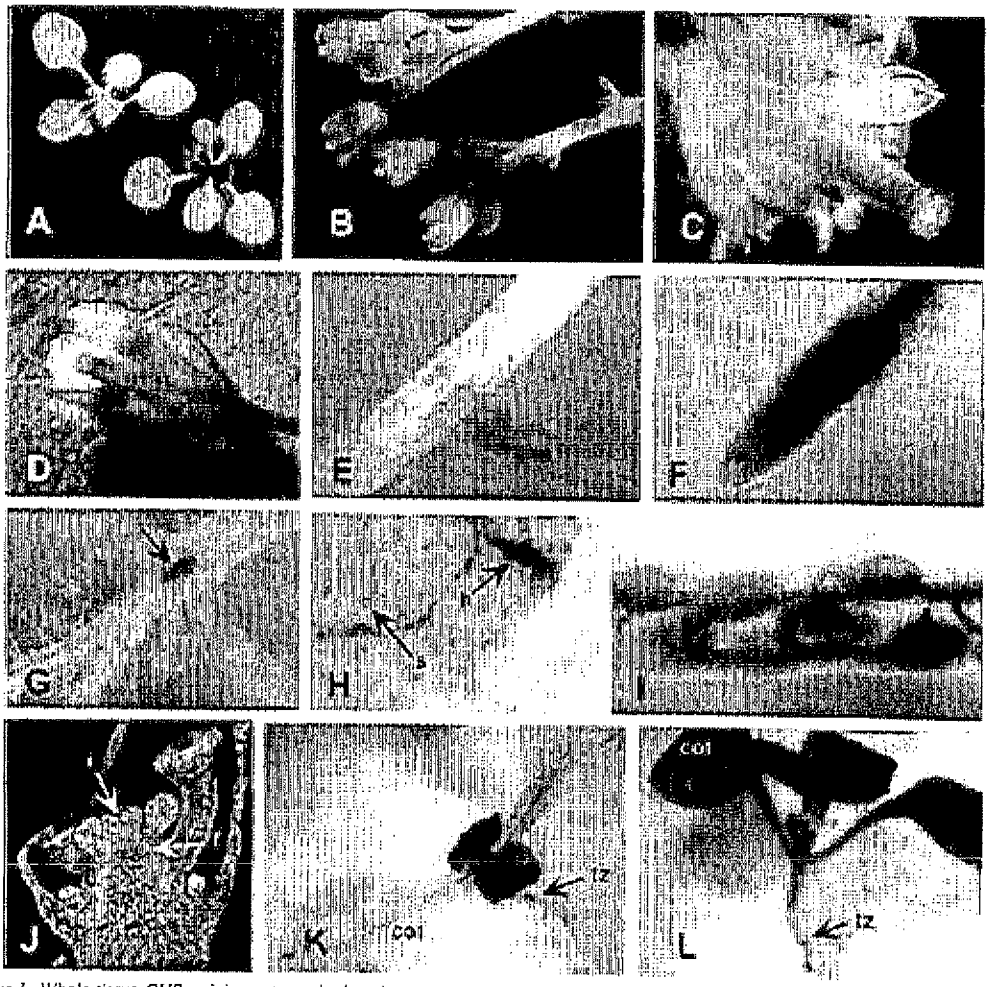

Figure 1. Whole-tissue GUS staining patterns in drought-stressed and control plants transformed with the *His1-3* GUS transgene. A. Plants homozygous for the transgene were sown directly in soil and seedlings were kept moist for 11 days after germination. Then, water was withheld from a subset of plants for seven days (well-watered control plant, top left, drought-stressed plant, bottom right). B. Inflorescences from drought-stressed (10 days without water) or well-watered 28-day old plants (well-watered control plant, top left; drought-stressed plant, bottom right). C. Flower from control 28-day-old plant. D. Flower from drought-stressed 28-day old plant. E. Secondary root from control 28-day old plant. F. Root apex from drought-stressed 28-day old plant. G. Junction between root and hypocotyl from a 7-day old seedling grown on MS medium supplemented with 1% sucrose. Transition zone indicated with arrow. H. Cotyledon from 7-day old seedling grown on 1% sucrose (h, hydathode, s, stomate). I. Early-stage 17 silique (8 mm in length), with valves removed to reveal replum and attached ovules, from well-watered 6-week old plant. J. Inflorescence meristem from well-watered 28-day old plant. Meristem is flanked by middle and late-stage two flower buds and stage five flower bud at right. (i, central initial cells; f, flank meristem). K. Seven-day old seedling grown on 1% sucrose (cot, cotyledon; tz, transition zone). L. Seven-day old seedling grown on 3% sucrose

Table 1. Transgene segregation and transcript levels in *35S::His1-3AS* lines.

Line	Kn^R/Kn^S[a]	*His1-3* transcript levels[b]
b1	15	++
b2	3	+
b4	3.5	+
b5	4	++
b6/9	3	++
b7	3	+++
b8	100%	+++
a1	3	+++
a2/3	10	|||
a4	8	+++

[a]Plants resistant/sensitive to kanamycin.
[b]Based on *His1-3* transcript levels in drought-stressed plants. RNA level ranging from very low (+) to wild-type levels (+++).

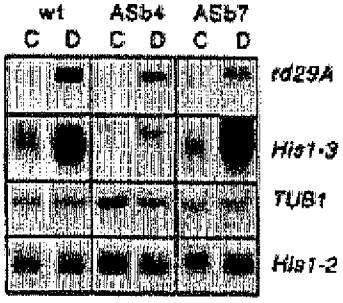

Figure 2. Steady-state transcript levels in shoots of soil-grown transgenic plant lines. Wild-type (wt) and antisense plant lines (ASb4 and ASb7) were either well watered (C) or subjected to drought stress (D). Total shoot RNA was then probed sequentially with the gene sequences indicated in the figure. *His1-3* transcripts were detected with an antisense-RNA probe (*TUB1* coding sequence detects transcripts of all β-tubulin isoforms).

To examine the effectiveness of the *35SHis1-3AS* transgene in reducing *His1-3* transcript levels throughout the plant, we performed RNA blot analyses on RNA extracted from seedling roots and shoots from wild-type plants and plants from each of the ten independent lines. Plants were grown in cell packs (40 cm^3/cell) under well-watered conditions and water was then withheld after two weeks. Pairwise comparisons of RNA levels in plants sharing the same limited root space were undertaken to insure that the plants were experiencing an equivalent degree of stress. Figure 2 shows the steady-state transcript levels for *His1-3* in addition to constitutively expressed and

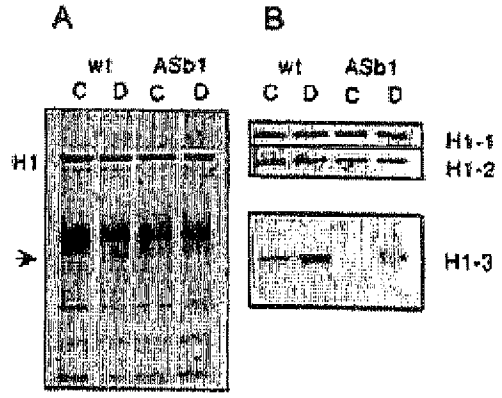

Figure 3. Protein blot analysis of a *35S::His1-3AS* plant line. A. Acid-extracted chromosomal proteins from well-watered (C) and drought-stressed (D) wild-type (wt) and antisense line b1 (ASb1) were separated on an 15% acid-urea polyacrylamide gel and stained with Coomassie blue. B. Proteins from a duplicate gel were transferred to nitrocellulose and probed sequentially with antibodies against the proteins indicated

drought-inducible genes in two representative lines. In wild-type plants, *His1-3* transcripts increase in shoots and roots upon progressive drought stress treatment as had been previously demonstrated (Ascenzi and Gantt, 1997). In shoots, concomitant increases are observed in the drought-inducible genes *Rd29A* and *AtDi21*. As expected, there is no change in the expression in the housekeeping genes encoding the β-tubulin isoforms or histone H1-2 (Figure 2). In contrast, *His1-3* is reduced in line ASb4 despite induction of the two other drought-inducible genes. However, not all of the transformed lines have reduced levels of *His1-3* transcripts. For example, ASb7 accumulates wild-type levels of *His1-3* (Figure 2). A summary of the degree of *His1-3* transcript reduction in shoots of the various lines is shown in Table 1. The relative induction in three antisense lines compared to wild-type was quantified by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). After eight days of progressive drought stress, *His1-3* transcript levels were 35%, 5% and 100% of wild type levels in ASb1 ($n = 4$), ASb4 ($n = 3$), and ASb7 ($n = 4$), respectively.

In order to evaluate the effectiveness of the antisense RNA at reducing protein levels *in planta*, we purified chromatin from leaves of drought-stressed wild-type and H1-3 antisense line ASb1. Proteins were extracted from chromatin with 0.2 M H_2SO_4 and subjected to acid-urea-PAGE (Figure 3A). In wild-type plants subjected to drought, a novel polypeptide is ob-

served within the range of expected mobility for H1-3 (indicated by an arrow). There was no difference in the intensity of staining in the band designated H1 (containing the major variants H1-1 and H1-2). In contrast, the putative H1-3 polypeptide was not detected in the drought-stressed antisense line. To identify the band as H1-3, we probed a blot from a duplicate gel with antibody against the N-terminus of H1-3 (Figure 3B). The polypeptide observed in the western blot has the same mobility as the faint band of lane 2 in Figure 3A. In wild-type plants, H1-3 was five-fold more abundant in chromatin from drought-stressed plants as compared to control plants. In antisense plants, H1-3 was undetectable in non-stress conditions and was decreased three-fold in drought-stressed plants relative to comparably stressed wild-type plants in good agreement with RNA-blot results. The amount of H1-1 and H1-2 extracted from chromatin did not differ between lines or treatments. We conclude that H1-3 is effectively reduced in a line expressing *35S::H1-3AS*, and that the transgene has no detectable effect on H1-1 and H1-2 levels. The amount of H1-3 in chromatin from the drought-stressed ASb1 line is only marginally higher than that found in unstressed wild-type plants, suggesting that drought-stressed *35S::His1-3AS* plants are unable to achieve a H1-3 level much greater than the uninduced wild-type level.

Plants overexpressing the His1-3 *cDNA contain increased levels of* His1-3 *transcripts and product*

His1-3 transcript levels were examined in three lines containing *35S::His1-3*. All lines exhibited increased *His1-3* transcripts relative to wild type (Figure 4 and data not shown). There was no change in the transcript levels of *His1-1* and *His1-2* in these plants (Figure 4 and data not shown). When the *35S::His1-3* line is subjected to drought, *His1-3* transcripts still accumulate to much higher levels than comparably drought-stressed control plants (Figure 4). We attribute the increased levels of *His1-3* in in drought-stressed *35S::His1-3* plants to be an additive effect of the transgene and endogenous gene. Alternatively, the stability of *His1-3* mRNA in in drought-stressed plants may be slightly enhanced, leading to increased steady-state message levels.

Next, we examined whether the additional *His1-3* transcripts were translated and incorporated into chromatin. Acid-extracted chromatin proteins from well-watered wild-type and a *35S::His1-3* line were subjected onto SDS-PAGE and blotted onto nitrocellulose (Figure 5). The blot was probed with antibodies against histones H4 and H1-3. As expected, little difference in the level of H4 was observed but a polypeptide with the mobility of H1-3 was observed only in the plants overexpressing *His1-3*. Histone H1-3 was nine times more abundant in the overexpressing line than in wild-type using a PhosphorImager with the H4 signal as the standard. This result demonstrates that unstressed plants containing *35S::His1-3* may achieve chromatin-bound H1-3 levels higher than stressed wild-type plants.

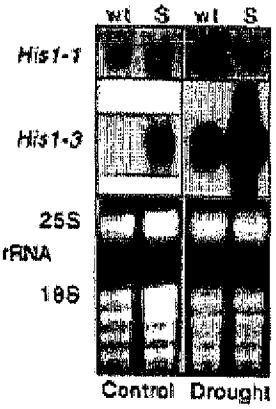

Figure 4. RNA blot analyses of a *35S::His1-3* plant line. Total RNA from wild-type (wt) and *35S::His1-3* line (S) was probed sequentially with labeled *His1-3* antisense RNA and *His1-1*. Treatment is shown below panels. Bottom panels show ethidium-bromide-stained gel before transfer.

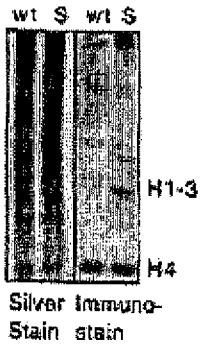

Figure 5. Protein blot analysis of a *35S::His1-3* plant line. Acid-extracted chromatin proteins from wild-type (wt) and *35SH1-3* line (S) were separated on a 12% SDS-polyacrylamide gel, transferred onto nitrocellulose and then probed simultaneously with anti-H1-3 and anti-bovine H4 (right panel). A duplicate gel was stained using a neutral silver stain method (left panel).

Table 2. Water status of drought-stressed wild-type and transgenic plants.

	n	RWC[a]	%WC
wt	17	81.1 ± 1.64	665 ± 28
ASb4[b]	8	84.5 ± 1.29	636 ± 42
ASb7	8	83.4 ± 1.92	674 ± 37
wt/ASb4[c]	8	95 ± 2.8	104 ± 11
wt/ASb7	8	98 ± 2.8	103 ± 5

[a]RWC and %WC are calculated as described in Materials and methods. All values are given as mean values (%) ± standard error. [b]Asb4 line is effective at reducing *His1-3* RNA while ASb7 is ineffective. [c]Ratio of wild-type to antisense line. Mean values of paired comparisons.

In order to test for autoregulation, we crossed plants homozygous for the *His1-3::GUS* construct to a plant homozygous for *35S::His1-3*. There was no difference in the GUS staining pattern or intensity suggesting that H1-3 does not regulate its own transcription (data not shown).

Plants expressing His1-3 *antisense transcripts do not have an altered water status during drought stress*

To determine if H1-3 performs a significant function related to the drought response, we examined whether plants with reduced levels of H1-3 had altered water relations. We did not observe differences between *35S::His1-3AS* and control plants under drought stress in terms of growth (leaf initiation, biomass, cessation of growth) during stress. Next, we estimated water deficit by measuring relative water content (RWC) and water content relative to dry weight (%WC). There was no significant difference between the water deficit of the effective ASb4 and ineffective ASb7 lines as estimated by both methods (Table 2). The ratios of water content between pairs of wild-type and antisense plants were examined, and again there was no difference between the ASb4 and ASb7 lines. These data suggest that the whole plant response to drought is unchanged in the ASb4 antisense line despite the demonstrated change in *His1-3* gene expression.

Plants misexpressing His1-3 *do not exhibit alterations in drought-responsive gene expression*

We measured the relative transcript levels of several drought-responsive and control genes in plants either overexpressing or underexpressing H1-3 during drought stress using plants grown as described previously. Plants that were experiencing late-stage stress or were grown under well-watered conditions (see Materials and methods) were harvested for these experiments. We examined four transgenic lines (one *35S::His1-3* line and three *35S::His1-3AS* lines) with varying degree of endogenous transcript suppression. The ratio of transcript levels in each pair, consisting of a transgenic plant and wild-type control, were calculated for each transcript. As a loading standard, we used *rps11* (Gantt and Thomson, 1990), a cytosolic ribosomal protein that appears not to respond to stress. We also examined the expression of non-drought-responsive genes *erpl15* (Thomson *et al.*, 1992), *His1-1*, *His1-2* (Gantt and Lenvik, 1991), and *TUB1* (Oppenheimer *et al.*, 1988) genes as controls. We selected the drought-inducible genes *AtDi21* (Gosti *et al.*, 1995), *Rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1993) and *rab18* (Lång and Palva, 1992) and the drought-repressible *cab2* (*AB165*) (Leutweiler *et al.*, 1986) and *rbcS-1A* (Krebbers *et al.*, 1988) genes. We did not observe consistent differences in any of the ten constitutively expressed or drought-responsive transcripts analyzed in multiple trials (data available upon request).

Discussion

Using transgenes, we have markedly altered the level of histone H1-3 in chromatin. We have not observed any changes in growth nor have we observed consistent changes in drought-responsive gene expression in plants with altered levels of the histone H1-3.

As a prelude to our histone modulation experiments, we examined the spatial expression of *His1-3* for two main reasons. We first compared the expression pattern of *His1-3* with *His1-1* and *His1-2* (Orcutt and Gantt, unpublished observations). This knowledge would help us interpret the results of the histone modulation experiments. If *His1-3* had an expression pattern distinct from *His1-1* and *His1-2* then it would be less likely that the genes have redundant functions. Second, knowledge of expression patterns would help us identify candidate genes whose overall gene expression could be affected by H1-3. In well-watered and drought-stressed *Arabidopsis* plants, reporter gene experiments suggest that *His1-3* is expressed primarily in expanding shoot tissues and in root meristems. Like *His1-1* and *His1-2*, expression does not seem to be cell-cycle-dependent in shoots because expression is not principally observed in the meristem. In

young seedlings, *His1-3* is expressed in hydathodes. These glands, located in the marginal serrations of leaves and tips of cotyledons, are thought to expel water from root pressure. The expression of some drought-inducible and sugar-inducible genes is also locally higher in these glands (e.g. Hua *et al.*, 1997; Martin *et al.*, 1997). Hua *et al.* (1997) described the developmental regulation of a drought-inducible gene encoding pyrroline-5-carboxylate reductase using the GUS reporter gene, the expression pattern of which in seedlings is nearly identical to *His1-3::GUS*. In addition to hydathodes, expression is observed in stomatal guard cells, root meristems and primordia, and the basal cell of trichomes. Martin *et al.* (1997) also observed that the sucrose-inducible patatin gene is developmentally expressed in what appears to be the transition zone. This is the same region in which we consistently observe *His1-3* expression. Interestingly, *His1-3::GUS* plants grown on high sucrose show increased reporter gene expression in hydathodes and other tissues. Increased *His1-3* transcript levels also occur in plants grown on 3% sucrose instead of 1%. This cannot be explained on the basis of an increase in osmotic potential (from −0.071 to −0.214 MPa) alone because 250 mM NaCl (−0.61 MPa) does not induce *His1-3* (Ascenzi and Gantt, 1997). Our findings describe a pattern of expression similar to what has been described for genes induced by water-stress and high levels of sucrose (Hua *et al.*, 1997; Martin *et al.*, 1997)

Analysis of linker histone function by modulating the amount of specific linker histone variants in animals has demonstrated a role for specific gene regulation by the different linker histones. Overexpression of H1° in a mouse cell line led to repression of the several transcripts tested while overexpression of the H1c variant had little or even the opposite effect on expression of the same genes (Brown *et al.*, 1996). The stimulatory effect of H1c overexpression on gene expression was attributed to its ability to promote a more open chromatin configuration, while the repression of gene expression by H1° was ascribed to its ability to be more readily incorporated in chromatin. Similarly, deletion of a gene encoding a single linker histone variant in a chicken cell line led to changes in the expression of several genes as evidenced by 2D-PAGE (Seguchi *et al.*, 1995). In another study using the same parental cell line, deletion of five of the six somatic histone H1s led to extensive changes in gene expression, but no change in growth rate (Takami and Nakayama, 1997). In a cell line with five deleted histone genes and hemizygous for the remaining histone H1 gene, fully one half the normal levels of H1 was bound to chromatin. Interestingly, the amount of HMG proteins was doubled. Studies using animals in which the levels of particular H1 variants have been modulated have generated contrasting results. In *Xenopus*, precocious expression of linker histones led to a premature loss of mesodermal competence whereas reduction of somatic linker histones by ribozymes extended the period that cells remained competent (Steinbach *et al.*, 1997). When the mouse gene encoding H1° was deleted by targeted disruption, the mutant animals exhibited a normal phenotype (Sirotkin *et al.*, 1995). Furthermore, the absolute amount of linker histone in the animals remained unchanged. From these studies, it is clear that linker histone variants regulate gene expression of a subset of genes and as a result regulate development in some cases. However, it appears that compensatory mechanisms abound that ensure the proper levels of linker proteins in chromatin.

Because linker histones have been shown to regulate the expression of a subset of genes in *Tetrahymena* and *Xenopus*, we examined the expression of several drought-responsive genes. We employed a candidate approach. Genes were selected that met the following criteria: (1) a change in expression occurs after *His1-3* induction during progressive drought stress; (2) spatial expression overlaps with *His1-3*, and (3) expression was repressed by drought. The third criterion is based on the observation that H1-3 binds more tightly to chromatin than either H1-1 or H1-2 and thus may be more likely to repress transcription (Ascenzi and Gantt, in press). Photosynthetic genes are thought to be repressed during drought stress to prevent photooxidation (Ingram and Bartels, 1996). In addition, it has been shown that *rbcS* (or clone pHH25) was dramatically down-regulated in *Arabidopsis* during rapid desiccation and drought stress (Baker *et al.*, 1994; Williams *et al.*, 1994). *RbcS-1A* and *-3B* have also been shown to be repressed by high 'nighttime' sucrose levels (Cheng *et al.*, 1998). We did not observe any consistent alteration in the levels of any of the transcripts tested. However, this does not prove that H1-3 does not modify the expression of genes or is entirely functionally redundant with H1-1 and H1-2 because we examined a limited number of genes. In addition, a transgenic approach may not be the ideal method for identifying the function of H1-3. A *His1-3* null mutant would insure that the protein is entirely absent from the plant, a condition we were not able to produce. A more thorough search for gene expression

phenotypes in *His1-3* transgenic plants and/or mutants should include 2D-PAGE, differential display and/or whole-genome analysis strategies.

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